QUANTITATIVE ASSAY FOR DETECTION OF NEWLY SYNTHESIZED RNA IN A CELL-FREE SYSTEM AND IDENTIFICATION OF RNA SYNTHESIS INHIBITORS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority from U.S. provisional application 60/555,765 filed January 6, 2003, which is hereby incorporated in its entirety.

BACKGROUND

[0002] HCV is one of the most prevalent causes of chronic liver disease in the United States, which accounts for about 15 percent of acute viral hepatitis, 60 to 70 percent of chronic hepatitis, and up to 50 percent of cirrhosis, end-stage liver disease, and liver cancer. Almost 4 million Americans, or 1.8 percent of the U.S. population, have antibodies to HCV (i.e., anti-HCV antibodies), indicating ongoing or previous infection with the virus. Hepatitis C causes an estimated 8,000 to 10,000 deaths annually in the United States. While the acute phase of HCV infection is usually associated with mild symptoms, some evidence suggests that only about 15% to about 20% of the infected people will clear HCV.

[0003] HCV is a small, enveloped, single-stranded positive strand RNA virus in the Flaviviridae family. The genome includes approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids. All of the protein products of HCV are produced by proteolytic cleavage of the polyprotein, carried out by one of three proteases: the host signal peptidase, the viral self-cleaving metalloproteinase (NS2), and the viral serine protease (NS3/4A). The combined action of these enzymes produces the structural proteins (C, E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) that are required for replication and packaging of the viral genomic RNA. NS5B is the viral RNA-dependent RNA polymerase (RDRP) that is responsible for the conversion of the input genomic RNA into a minus strand copy (complimentary RNA, or cRNA); the cRNA then serves as a template for transcription by NS5B of the positive sense genomic/messenger RNA. The HCV replicase is the complex of proteins that are necessary for the accurate and efficient synthesis of viral replicon RNA.

[0004] Currently, the only effective therapy against HCV is alpha-interferon, which reduces the amount of virus in the liver and blood (e.g., viral load) in only a small proportion

of infected patients. Standard forms of interferon, however, are now being replaced by pegylated interferons (peginterferons), alpha interferons that have been modified chemically by the addition of a large inert molecule of polyethylene glycol. At the present time, the optimal regimen including interferon appears to be a 24- or 48-week course of a combination of pegylated alpha interferon and the nucleoside ribavirin, an oral antiviral agent that has activity against a broad range of viruses. Nonetheless, response rates to the combination interferon/ribavirin therapy may be moderate for certain HCV genotypes, i.e., a response rate of about 50% to about 60%, although response rates for selected genotypes of HCV (notably genotypes 2 and 3) are typically higher. Another drawback to the current therapy is that there are often significant adverse side effects associated with each of these agents including, for example, flu-like symptoms; bone marrow suppressive effects; neuropsychiatric effects such as marked irritability, anxiety, personality changes, depression, and even suicide or acute psychosis; histamine-like side effects; and anemia.

[0005] Taken together, the preceding facts indicate a significant need for effective small molecule inhibitors of HCV replication that do not suffer from the above-mentioned drawbacks. A particularly useful class of inhibitors of HCV, as well as other positive strand RNA viruses, is inhibitors of viral RNA synthesis.

[0006] While accurate and efficient assays for identifying HCV RNA synthesis inhibitors may be useful tools for identifying effective small molecule HCV therapeutics, no such system has been developed. An *in vitro* replication assay using recombinant NS5B polymerase has been reported. However, in this system, the purified form of NS5B polymerase lacked template specificity and produced various lengths of RNA products. These phenomena are very different from HCV RNA replication *in vivo*. To better reflect the HCV RNA replication process in the cell, a cell-free HCV replication system was established using whole cell lysates or membrane fractions of cells expressing the HCV replicon. In this cell-free system, radioactive P^{32}-UTP or P^{32}-CTP was used to label newly synthesized HCV RNA, and then the reaction products were resolved by gel electrophoresis, followed by autoradiography. Because these assays require gel electrophoresis to separate the full length HCV RNA from other RNA molecules, the results are difficult to quantify, often inaccurate, and poorly reproducible. Additionally, while it appears to be clear that RNA elongation occurs in this cell-free system, there is no convincing evidence that *de novo* RNA initiation occurs in this system.

SUMMARY

[0007] An assay for detecting newly synthesized viral RNA in a cell-free system, and for identifying compounds that inhibit positive strand RNA viruses such as the Hepatitis C Virus (HCV) is provided herein.

[0008] Provided herein is an assay for detecting newly synthesized RNA from a positive strand RNA virus, such as HCV virus, which is more efficient and quantitative than previously reported assays. This assay is useful for identifying inhibitors of RNA synthesis of a positive strand RNA virus, including inhibitors of HCV RNA synthesis. Certain embodiments of the assay include methods for distinguishing RNA synthesis initiation inhibitors from RNA elongation inhibitors.

[0009] Provided herein is a method for determining whether a test compound inhibits RNA synthesis of a positive strand RNA virus. The method comprises:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, a labeled nucleotide analog, and the test compound, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

detecting the newly synthesized RNA population comprising the labeled nucleotide analog;

quantitating the newly synthesized RNA population comprising the labeled nucleotide analog to provide a test RNA amount; and

comparing the test RNA amount with a control RNA amount of a control newly synthesized RNA population comprising the labeled nucleotide analog produced in the absence of the test compound, wherein a decrease in the test RNA amount compared to the control RNA amount indicates that the test compound inhibits RNA synthesis of the positive strand RNA virus.

[0010] Further provided herein is a method for quantitating newly initiated RNA of a positive strand RNA virus comprising:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, and a labeled nucleotide analog, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

hybridizing a probe and the newly synthesized RNA population comprising the labeled nucleotide analog, under stringent hybridization conditions, wherein the probe is complementary to at least a portion of a transcription initiation region of the newly synthesized RNA population;

digesting unhybridized, single-stranded RNA with a single-strand specific ribonuclease to form a protected RNA population comprising the labeled nucleotide analog;

detecting the protected RNA population comprising the labeled nucleotide analog; and

quantitating the protected RNA population comprising the labeled nucleotide analog.

[0011] Also provided is a method for determining whether a test compound is an RNA synthesis initiation inhibitor of a positive strand RNA virus comprising:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, a labeled nucleotide analog, and the test compound, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

hybridizing a probe and the newly synthesized RNA population comprising the labeled nucleotide analog, under stringent hybridization conditions, wherein the probe is complementary to at least a portion of an initiation region of the newly synthesized RNA population;

digesting unhybridized, single-stranded RNA with a single-strand specific ribonuclease to form a protected RNA population;

detecting a protected RNA population comprising the labeled nucleotide analog;

quantitating the protected RNA population comprising the labeled nucleotide analog to provide a test RNA amount; and

comparing the test RNA amount with a control RNA amount of protected RNA comprising the labeled nucleotide analog but produced in the absence of the test compound, wherein a decrease in the test RNA amount compared to the control RNA amount indicates that the test compound inhibits RNA synthesis initiation of the positive strand RNA virus.

BRIEF DESCRIPTION OF THE FIGURES

[0012] Figure 1 shows a schematic of the inhibition of HCV replicon replication by transcription initiation and elongation inhibitors.

[0013] Figure 2 shows an autoradiogram illustrating use of an RNA synthesis inhibitor to demonstrate *de novo* initiation of RNA synthesis in isolated replication complexes.

DETAILED DESCRIPTION

[0014] Prior to setting forth the invention in detail, it may be helpful to provide definitions of certain terms to be used herein. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs.

TERMINOLOGY AND MOLECULAR DESCRIPTION

[0015] A "replicon" as used herein includes a genetic element, for example, a plasmid, cosmid, bacmid, phage or virus that is capable of replication largely under its own control. A "replicon RNA" includes RNA produced by transcription of a replicon, e.g., a double-stranded DNA replicon. A replicon may be either RNA or DNA, and may be single or double-stranded. A suitable replicon is a replicon from a single-stranded positive strand RNA virus such as a virus of the Picornaviridae family, the Calciviride family, the Togaviridae family, the Coronaviridae family, or the Flaviviridae family. Viruses of the Flaviviridae family include, for example, Hepatitis C Virus, West Nile Virus, Dengue Virus, Kunjuin Virus, Yellow Fever Virus, Bovine Viral Diarrhea Virus, Tick Born Encephalitis Virus, Japanese Encephalitis Virus, and Venezuelan Equine Encephalitis Virus. In one embodiment, the replicon is a Hepatitis C Virus replicon.

[0016] In positive strand RNA viruses, replication is performed by a multi-protein-RNA complex called a "replicase complex". As used herein, a "replicase complex" is an active complex of polypeptides and RNA which is capable of complete and accurate viral replicon RNA synthesis under cell-free conditions suitable for viral RNA replication. By complete and accurate viral replicon RNA synthesis, it is meant that the replicase complex is capable of producing full-length viral replicon RNAs. In addition, the isolated replicase complex should show specificity for replication of the replicase RNA of the corresponding

positive strand RNA virus. In one embodiment, an isolated replicase complex comprises a viral replicon template RNA for the positive strand RNA virus. An "isolated replicase complex" is a replicase complex which has been removed from its natural cellular environment, such as a cell expressing a viral replicon RNA. "Isolated replicase complex" includes the membrane fraction of a cell expressing the viral replicase RNA. The isolated replicase complex may be separated from the cell nucleus, chromosomal DNA, and cytoplasmic materials, for example. In addition, an isolated replicase complex may comprise one or more polypeptides expressed from a recombinant expression system, so long as the complex is capable of complete and accurate viral replicon RNA synthesis. The replicase complex of HCV, for example, includes the NS5B protein which has RNA-dependent RNA polymerase activity, and other protein factors

[0017] "Nucleic acid" or a "nucleic acid molecule" refers to a DNA or RNA molecule, either single or double-stranded and, if single-stranded, the molecule of its complementary sequence in either linear or circular form. A sequence or structure of a particular nucleic acid molecule can be described according to the normal convention of providing the sequence in the 5' to 3' direction.

[0018] The term "isolated nucleic acid molecule" includes nucleic acid molecules that are separated from an intact cellular environment. An "isolated nucleic acid molecule" may be, for example, a template RNA that is separated from the cell nucleus, chromosomal DNA, and other cellular materials which are not membrane-associated. Moreover, an "isolated" nucleic acid molecule, such as a viral replicon template RNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In some embodiments, an isolated viral replicon template RNA may be purified as a portion of a membrane fraction of a cell expressing a viral replicon RNA. Such a membrane fraction may also comprise isolated replicase complexes. Substantially free of other cellular material includes, for example, a cellular fraction such as, for example, a membrane bound fraction. By substantially free of other cellular material, it is meant that an isolated nucleic acid molecule may be greater than or equal to about 70%, about 75%, about 80%, about 85%, about 90%, about 95% or about 99% free of unwanted cellular materials, such as, for example, components of cellular fractions other than the membrane-bound fraction.

[0019] In some embodiments, an "isolated" nucleic acid may be free of sequences

which naturally flank the nucleic acid (i.e., sequences located at the 5' and/or 3' ends of the nucleic acid) in the genomic DNA or RNA of the organism from which the nucleic acid is derived. For example, the isolated nucleic acid molecule may contain less than about 5 kb, about 4 kb, about 3 kb, about 2 kb, about 1 kb, about 0.5 kb or about 0.1 kb of 5' and/or 3' nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. In this sense, an isolated nucleic acid may be, for example, a DNA vector encoding a viral replicon RNA which has been purified by standard DNA purification methods.

[0020] "Natural allelic variants", "mutants" and "derivatives" of particular sequences of nucleic acids refer to nucleic acid sequences that are closely related to a particular sequence but which may possess, either naturally or by design, changes in sequence or structure. By closely related, it is meant that greater than or equal to about 75%, but often, greater than or equal to about 90%, of the nucleotides of the sequence match over the defined length of the nucleic acid sequence. Changes or differences in nucleotide sequence between closely related nucleic acid sequences may represent nucleotide changes in the sequence that arise during the course of normal replication or duplication in nature of the particular nucleic acid sequence. Other changes may be specifically designed and introduced into the sequence for specific purposes, such as to change an amino acid codon or sequence in a regulatory region of the nucleic acid. Such specific changes may be made *in vitro* using a variety of mutagenesis techniques or produced in a host organism placed under particular selection conditions that induce or select for the changes. Such sequence variants generated specifically may be referred to as "mutants" or "derivatives" of the original sequence.

[0021] Different "variants" including "natural allelic variants" of, for example, the HCV genome exist in nature. These variants may be alleles characterized by differences in the nucleotide sequences of the gene coding for a protein, or may involve different RNA processing or post-translational modifications. The skilled person can produce variants having single or multiple amino acid substitutions, deletions, additions or replacements. These variants may include inter alia: a) variants in which one or more amino acids residues are substituted with conservative or non-conservative amino acids, b) variants in which one or more amino acids are added, and c) variants in which one or more amino acids include a substituent group.

[0022] "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., atg) in front of a protein-encoding gene, splicing signals for introns (if introns are present), maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter. By "promoter" is meant minimal sequence sufficient to direct transcription. Also included are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters are included. A replicon may include operably linked expression control sequences.

[0023] The term "probe" as used herein refers to an oligonucleotide or polynucleotide comprising either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be, for example, a single-stranded RNA transcribed *in vitro* from a DNA template. A probe may be either single-stranded or double-stranded, and in one embodiment is single-stranded. The exact length of the probe will depend upon many factors, including the temperature, the source of the probe, and use. The probes are selected to be "substantially" complementary to a strand of a particular target nucleic acid sequence. This means that the probes are sufficiently complementary so as to be able to "specifically hybridize" or anneal with their

respective target strands under a set of pre- determined conditions. The term specifically hybridize means that the probe has a greater probability of hybridizing to its target sequence than other non-target sequences.

[0024] Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. A small (e.g., less than or equal to about 10%) of mismatched nucleotides in the primer and the target sequence is also permissible.

[0025] The term "oligonucleotide" is defined as a nucleic acid molecule comprising two or more ribonucleotides or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide. In one embodiment, an oligonucleotide comprises less than 100 nucleotides, more specifically less than or equal to about 50 nucleotides, and most specifically less than or equal to about 30 nucleotides.

[0026] A "polynucleotide" probe or primer specifically comprises less than or equal to about 1000 nucleotides, more specifically less than or equal to about 800 nucleotides, and most specifically less than or equal to about 500 nucleotides. A polynucleotide primer also specifically comprises greater than or equal to about 100 nucleotides, more specifically greater than or equal to about 150 nucleotides, and most specifically greater than or equal to about 200 nucleotides.

[0027] "Stringency conditions" for hybridization is a term of art which refers to the incubation and wash conditions, e.g., conditions of temperature and buffer concentration, which permit hybridization of a first nucleic acid to a second nucleic acid; the first nucleic acid may be perfectly (i.e., 100%) complementary to the second, or the first and second may share some degree of complementarity which is less than perfect (e.g., 70%, 75%, 85%, 95%, 98%). For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity.

[0028] "High stringency conditions", "moderate stringency conditions" and "low stringency conditions" for nucleic acid hybridizations are explained in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, "*Current Protocols in Molecular Biology*", John Wiley & Sons, (1998)). The exact conditions which determine the stringency of hybridization depend not only on ionic strength (e.g., 0.2X SSC, 0.1X SSC), temperature

(e.g., room temperature, 42°C, 68°C) and the concentration of destabilizing agents such as formamide or denaturing agents such as SDS, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. SSC buffer is 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0. High, moderate or low stringency conditions can be determined empirically.

[0029] By varying stringency conditions for hybridization from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize (e.g., selectively) with the most similar sequences in the sample can be determined.

[0030] Exemplary conditions are described in Krause, M.H. and S.A. Aaronson, *Methods in Enzymology, 200*:546-556 (1991). Also, in, Ausubel, *et al.*, "*Current Protocols in Molecular Biology*", John Wiley & Sons, (1998), which describes the determination of washing conditions for moderate or low stringency conditions. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Starting from the lowest temperature at which only homologous hybridization occurs, each °C by which the final wash temperature is reduced (holding SSC concentration constant) allows an increase by about 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in T_m of about 17°C. Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought.

[0031] For example, a low stringency wash can comprise washing in a solution containing 0.2X SSC/0.1% sodium dodecyl sulfate (SDS) for 10 minutes at room temperature; a moderate stringency wash can comprise washing in a prewarmed solution (42°C) solution containing 0.2X SSC/0.1% SDS for 15 minutes at 42°C; and a high stringency wash can comprise washing in prewarmed (68°C) solution containing 0.1X SSC/0.1% SDS for 15 minutes at 68°C. Furthermore, washes can be performed repeatedly or sequentially to obtain a desired result as known in the art. Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of identity or similarity between the target nucleic acid molecule and the primer or probe used.

[0032] A "test compound" as defined herein refers to a chemical, nucleic acid, polypeptide, amino acid, or other compound which is to be tested. Examples of test compounds include, but are not limited to, drug candidates, such as derived from arrays of small molecules generated through general combinatorial chemistry, as well as any other substances thought to have potential biological activity.

DESCRIPTION OF THE ASSAYS

[0033] Provided herein is a method for determining whether a test compound inhibits RNA synthesis of a positive strand RNA virus. The method comprises:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, a labeled nucleotide analog, and the test compound, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

detecting the newly synthesized RNA population comprising the labeled nucleotide analog;

quantitating the newly synthesized RNA population comprising the labeled nucleotide analog to provide a test RNA amount; and

comparing the test RNA amount with a control RNA amount of a control newly synthesized RNA population comprising the labeled nucleotide analog produced in the absence of the test compound, wherein a decrease in the test RNA amount compared to the control RNA amount indicates that the test compound inhibits RNA synthesis of the positive strand RNA virus.

[0034] Further provided herein is a method for quantitating newly initiated RNA of a positive strand RNA virus comprising:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, and a labeled nucleotide analog, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

hybridizing a probe and the newly synthesized RNA population comprising the labeled nucleotide analog, under stringent hybridization conditions, wherein the probe is complementary to at least a portion of a transcription initiation region of the newly synthesized RNA population;

digesting unhybridized, single-stranded RNA with a single-strand specific ribonuclease to form a protected RNA population comprising the labeled nucleotide analog;

detecting the protected RNA population comprising the labeled nucleotide analog; and

quantitating the protected RNA population comprising the labeled nucleotide analog.

[0035] Also provided is a method for determining whether a test compound is an RNA synthesis initiation inhibitor of a positive strand RNA virus comprising:

contacting an isolated replicase complex for the positive strand RNA virus, an isolated viral replicon template RNA for the positive strand RNA virus, a labeled nucleotide analog, and the test compound, under conditions sufficient for *in vitro* RNA synthesis, to form a newly synthesized RNA population comprising the labeled nucleotide analog;

hybridizing a probe and the newly synthesized RNA population comprising the labeled nucleotide analog, under stringent hybridization conditions, wherein the probe is complementary to at least a portion of an initiation region of the newly synthesized RNA population;

digesting unhybridized, single-stranded RNA with a single-strand specific ribonuclease to form a protected RNA population;

detecting a protected RNA population comprising the labeled nucleotide analog;

quantitating the protected RNA population comprising the labeled nucleotide analog to provide a test RNA amount; and

comparing the test RNA amount with a control RNA amount of protected RNA comprising the labeled nucleotide analog but produced in the absence of the test compound, wherein a decrease in the test RNA amount compared to the control RNA amount indicates that the test compound inhibits RNA synthesis initiation of the positive strand RNA virus.

[0036] In some embodiments of the above methods, the isolated viral replicase complexes and the isolated viral replicon template RNA are provided by transfecting a cell line with an isolated DNA template for a viral replicon or an isolated viral RNA, to provide a transfected cell line, incubating the transfected cell line under conditions suitable for viral replication, and isolating replicase complexes and viral replicon template RNA from the cell membrane fraction of the transfected cell line.

[0037] In other embodiments of the above methods, the isolated viral replicase complexes and isolated viral replicon template RNAs may be provided by acutely infected or

persistent infected primary hepatocytes, lymphocytes or other cell lines, incubating the infected cell line under conditions suitable for viral replication, and isolating the replicase complexes comprising viral replicon RNA from the cell membrane fraction. The replicase complexes may be isolated from infected primary cells or cell lines.

[0038] In certain embodiments of the methods described herein, the positive strand RNA virus is Hepatitis C Virus, and a suitable DNA template for the HCV viral replicon is, for example, SEQ ID NO: 1, which can also be described as GenBank Accession No. AJ242652. Other suitable replicon template DNAs include, for example, AB114136, AJ242654, AJ242653, and AJ242651 (SEQ ID NOs. 2-5).

[0039] In certain embodiments of the methods, the labeled nucleotide analog is an analog capable of being recognized by a specific antibody, an analog which can be recognized via a high specificity binding reaction, or an analog directly detectable as a result of a physical property of the analog. In some embodiments, the labeled nucleotide analog is an analog capable of being recognized by a specific antibody, such as 5'-bromouridine 5'-triphosphate (Br-UTP). In other embodiments, the labeled nucleotide analog is a radioactively labeled nucleotide.

[0040] In the methods described herein, detection of a newly synthesized RNA population may be accomplished by contacting a newly synthesized RNA population with an antibody specific for the labeled nucleotide analog, and immuno-precipitating the newly synthesized RNA population comprising the labeled nucleotide analog to form an immuno-precipitated RNA population. In some embodiments, the nucleotide analog is Br-UTP, and the specific antibody is an anti-BrdU antibody. The methods described herein include quantitating the newly synthesized RNA population by performing real time PCR on the immuno-precipitated RNA population. In other embodiments, detection of a newly synthesized RNA population may be accomplished by detecting a radioactively labeled nucleotide by, for example, non-denaturing gel electrophoresis followed by autoradiography.

[0041] In some embodiments, a 2'-O-methylated nucleotide may be employed during the replication reaction to increase the yield of newly synthesized RNA.

[0042] In order to form a newly synthesized RNA population, an isolated replicase complex and isolated viral replicase RNA template are employed. The isolated viral replicon template RNA comprises a transcription initiation region. The template RNA may be, for example, isolated as a component of the replicase complex. In some embodiments, an isolated viral template RNA may be added to the replicase complex.

[0043] A viral replicon RNA or DNA vector encoding the viral replicon RNA may be transfected into a cell line suitable for expression of the viral replicon RNA. Suitable cell lines include, for example, mammalian cell lines such as Vero cells, HeLa cells, CHO cells, COS cells, WI38 cells, N1H-3T3 cells (and other fibroblast cells, such as MRC-5 cells), MDCK cells, KB cells, SW-13 cells, MCF7 cells, BHK cells, HEK-293 cells, HepG2 cells, Bowes melanoma cells cell lines; and chicken embryonic fibroblast (CEF) cell lines. In one embodiment, the cell line is a human cell line, such as, for example, a human hepatosoma cell line such as Huh-7. Suitable means of transfection include, for example, calcium phosphate co-precipitates, mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, as well as other techniques known in the art.

[0044] A suitable DNA vector for production of an HCV replicon RNA, for example, comprises, 5'-3', the HCV-IRES, the neomycin phosphotransferase (neo) gene, the IRES of encephalomyocarditis virus, which directs translation of HCV sequences NS3 to NS5B, and the 3'-NTR. The sequence of a DNA vector suitable for production of the HCV replicon RNA has been deposited in GenBank (Accession no. AJ242652) (SEQ ID NO:1). The HCV replicon DNA vector may be transfected into Huh-7 cells by electroporation. HCV replicon RNA may be transcribed *in vitro* using either SP6 RNA polymerase or T7 RNA polymerase, for example, from a plasmid DNA comprising the appropriate promoter with the cDNA encoding the replicon RNA downstream of the promoter. The DNA template for production of a viral replicase RNA may be produced by PCR amplification using a DNA template encoding the viral replicon and specific primers one of which comprises an SP6 or T7 RNA polymerase promoter. Alternatively, the DNA template for production of a viral replicase RNA may be produced by reverse transcription of the viral replicase RNA using primers one of which comprises an SP6 or T7 RNA polymerase promoter.

[0045] Once the viral replicon RNA or DNA vector encoding the viral replicon RNA is transfected into a cell, the cell culture may be grown to confluency. The cells in the culture produce replicase complexes comprising viral replicon template RNAs, which can then be isolated from the cells. Viral replicon RNA includes minus strand viral replicon template RNA, and optionally positive strand RNA.

[0046] Alternatively, infected cells such as acutely infected or persistent infected primary hepatocytes, lymphocytes or other cell lines may be employed to isolated viral replicon RNA. Infected primary cells or cell lines may be employed. The infected cell line is incubated under conditions suitable for viral replication, and the replicase complexes comprising viral replicon RNA are isolated from the cell membrane fraction.

[0047] In order to isolate replication complexes and viral replicon template RNA, the cells (i.e., transfected or infected with a viral replicon) may be lysed and centrifuged at low speed (e.g., about 900 X to about 1000 X g) to remove cellular debris. The supernatant may then be centrifuged at higher speed (e.g., about 15000 X g) to obtain the membrane fraction, which comprises isolated viral replicon complexes and optionally comprises viral replicon template RNA. It has been shown that replication of the HCV RNA occurs in a membrane-bound replication complex. Thus, HCV replicon complexes and replicon template RNA can be isolated by isolating the membrane fraction of the cells expressing the HCV replicon RNA.

[0048] The isolated membrane fraction containing replicase complexes (e.g., an aliquot of the membrane fraction of a cell line expressing a viral RNA replicon) and isolated replicon template RNA are used to perform RNA replication *in vitro* in a cell-free system. The isolated replicase complexes and isolated viral replicon template RNA are contacted with a labeled nucleotide analog under conditions sufficient to promote *in vitro* RNA synthesis. Conditions sufficient to promote *in vitro* RNA synthesis include suitable buffers, and nucleoside triphosphates (i.e., ATP, UTP, CTP, and GTP). The labeled nucleotide analog is incorporated into a newly synthesized RNA population, and provides a means for detecting only the newly synthesized RNA, and not the template RNA. In one embodiment, suitable conditions for replication include, for example, incubation in 50 μl (total volume) of 50 mM HEPES (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 50 units RNasin, 10 μg/ ml actinomycin D, 2.5 mM ATP, 0.5 mM each CTP, GTP, labeled UTP and an aliquot of a membrane fraction of a cell line expressing the viral replicon RNA, e.g., isolated replicase complexes and isolated

viral replicon template RNA. In another embodiment, suitable replication conditions include 50 mM HEPES (pH 7.3); 10 mM KCl; 10 mM $MgCl_2$; 0.3 mM $MnCl_2$; 20 Units of RNAse inhibitor; 10 μg of actinimycin D per mL; 0.5 mM ATP,GTP, and UTP; 10 μCi of [α-P^{32}] CTP; and 6 μl of the membrane fraction of a cell line expressing the viral replicon RNA in a total volume of 60 μl. The replication reaction is continued for a time sufficient to produce a desired amount of newly synthesized RNA.

[0049] In some embodiments, the replication conditions may include adding a 2'-O-methylated nucleotide such as, for example, 2'-O-methyl-5-methyluridine-5'- triphosphate to the replication mixture. The inclusion of a 2'-O-methylated nucleotide such as 2'-O-methyl-5-methyluridine-5'triphosphate may increase the efficiency of the replication complex, resulting in an increase in the amount of labeled product obtained in the absence of the 2'-O-methyl-5-methyluridine-5'- triphosphate. Without being held to a particular theory, it is believed that a 2'-O-methylated nucleotide, particularly 2'-O-methyl-5-methyluridine-5'-triphosphate increases the efficiency of the replicase complex when a radiolabeled nucleotide such as, for example, P^{32}-CTP is added to the reaction at low concentrations.

[0050] Suitable labeled nucleotide analogs include, for example, analogs which are capable of being recognized by specific antibodies (e.g., Br-UTP), analogs such as a biotin labeled nucleotide (e.g., biotin-CTP) which can be recognized via a high specificity binding reaction (i.e., biotin/avidin or biotin/streptavidin binding), and analogs which are directly detectable as a result of a physical property of the analog, such as radioactivity (e.g., a P^{32}-labeled nucleotide), fluorescence, luminescence etc., for example a fluorescein-nucleotide (e.g., fluorescein-UTP). In one embodiment, the labeled nucleotide analog is 5'-bromouridine 5'-triphosphate (Br-UTP). The RNA containing Br-UTP may be isolated by immuno-precipitation and/or detection with an anti-BrdU antibody. The anti-BrdU antibody may optionally be labeled with a tag suitable for direct detection of the antibody such as, for example, fluorescein, or other dyes such as those available from Molecular Probes. In other cases, the antibody may be detected via binding to a labeled secondary antibody such as, for example, a rabbit or mouse IgG labeled with alkaline phosphatase or horseradish peroxidase.

[0051] The labeled nucleotide analog is added to the replication mixture so that the labeled nucleotide analog is incorporated into the newly synthesized RNA (e.g., newly synthesized viral replicon RNA) in place of at least a portion of the corresponding unlabeled

nucleotide. For example, Br-UTP may be used in place of at least a portion of the UTP. In certain cases, all of the corresponding nucleotide may be replaced with the labeled nucleotide analog. After replication, there are two populations of RNA (e.g., viral replicon RNA): an unlabeled RNA population (i.e., the RNA population present before replication) and a labeled RNA population (e.g., the newly synthesized RNA population). The newly synthesized RNA population, which contains the labeled nucleotide analog, can be distinguished from the RNA population which was present before the replication step, because the RNA population which was present before replication will not contain the labeled nucleotide analog. Because the newly synthesized RNA can be distinguished from the RNA present prior to replication, the amount of newly synthesized RNA (e.g., viral replicon RNA) can be detected and/or quantified by employing a means of RNA detection and/or quantitation. The means for RNA detection and quantitation may be the same or different.

[0052] After replication, the RNA in the replication mixture optionally may be purified using, for example, a commercially available RNA purification kit. The newly synthesized RNA is then detected using a means for detecting the RNA. Detection preferably comprises quantitative detection. The method for detecting the RNA is selected based on the labeled nucleotide analog employed. For example, in the case of an analog capable of being recognized by specific antibodies, a specific antibody may be employed to immuno-precipitate the labeled RNA. The immuno-precipitated RNA may be detected, for example, by directly detecting a fluorescent tag present on the antibody. Alternatively, the immuno-precipitated RNA may be detected with a labeled secondary antibody. In another alternative, the immuno-precipitated labeled RNA can be amplified and detected using real-time PCR.

[0053] For example, a newly synthesized RNA population containing Br-UTP as the labeled nucleotide analog may be immuno-precipitated using an anti-BrdU monoclonal antibody. The unlabeled, template RNA will not be precipitated, while the newly synthesized Br-UTP-labeled RNA will be specifically precipitated. The immuno-precipitated, labeled RNA may then be quantified using real time PCR. Thus, in the case of, for example, a Br-UTP labeled viral replicon RNA precipitated with anti-BrdU antibody, the method for detection comprises contacting the population of viral replicon RNAs with an anti-BrdU antibody, precipitating the viral replicon RNAs, and detecting and quantitating the labeled viral replicon RNAs using a suitable method such as, for example, real time PCR.

[0054] Real time PCR (polymerase chain reaction) is a quantitative reverse transcription-PCR reaction (RT-PCR). Early in RT-PCR, reagents are in excess, template and product are at low enough concentrations that product renaturation does not compete with primer binding, and amplification proceeds at a substantially constant, exponential rate. At some variable point during the reaction, the reaction rate ceases to be exponential, and enters a linear phase of amplification. Late in the amplification cycle, little product is made. Real time PCR allows the collection of data during the exponential phase of amplification, and thus allows for accurate quantitation of the amount of amplified product. Detection and quantitation of the double-stranded DNA produced in the PCR reaction is done using a fluorescent reporter, the signal of which increases in direct proportion to the amount of double-stranded DNA PCR product in a reaction. Taqman® probes labeled with fluorescent dye such as FAM (6-carboxy-fluorescein) may be used. Another suitable reporter is the double-strand DNA-specific dye SYBR® Green (Molecular Probes). SYBR® Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. Other alternatives reporters include hybridization probes which use fluorescence energy transfer (FRET) for detection.

[0055] When the labeled nucleotide analog is a radioactive nucleotide such as P^{32}-CTP, the labeled RNAs may be detected by non-denaturing gel electrophoresis followed by autoradiography, direct radioactive detection such as Phosphorimaging, and the like.

[0056] In the case of a labeled nucleotide analog which can be recognized via a high specificity binding reaction, the labeled RNAs may be isolated by employing the high specificity binding reaction. For example, a biotin-labeled nucleotide can be recognized by avadin or streptavadin. Once the labeled RNAs have been isolated, the labeled RNAs can be amplified and detected using real-time PCR. In the case of a directly detectable nucleotide analog, the labeled nucleotide analog can be directly detected using fluorescence, luminescence, and the like.

[0057] Suitable controls for quantitation of the newly synthesized RNA population follow. Template RNAs isolated from cells untransfected with a DNA vector or viral RNA and subjected to replication, have no detectable amount of newly synthesized RNA. Template RNAs isolated from cells transfected with a template DNA vector or viral RNA, but replicated without the labeled nucleotide analog, have only background levels of newly synthesized RNA. Template RNAs from cells transfected with template DNA vector or viral

RNA, replicated in the presence of a labeled nucleotide analog, which are not detected with the means for detection, show only background levels of newly synthesized RNA. Template RNAs from cells transfected with template DNA vector or viral RNA, replicated in the presence of labeled nucleotide analog and detected with the means for detection should, however, show a strong newly synthesized RNA signal in this assay.

[0058] The assay may be employed to quantitate a newly initiated RNA population. In the case of HCV, for example, most (e.g., about 90%) of the newly synthesized viral RNAs are the elongation products of previously initiated (i.e., initiated) template RNAs. In an initiated template RNA, a portion of the newly synthesized RNA is previously made using a minus strand template. Upon addition of the appropriate factors (e.g., buffers and nucleoside triphosphates), elongation of the initiated RNA can proceed to form the full-length RNA. Only a small fraction (e.g., less than about 10%) of the newly synthesized RNAs represent newly initiated products formed from uninitiated template RNAs (i.e., pre-initiation). Both the previously initiated and newly initiated RNAs will comprise the labeled nucleotide analog in the elongation region of the RNA. Only the newly initiated RNAs, however, will comprise the labeled nucleotide analog in the transcription initiation region. A method to select and quantitate newly initiated RNAs comprises employing RNA protection to the newly synthesized RNA population which may comprise a previously initiated RNA population as well as newly initiated RNAs.

[0059] In the RNAse protection assay, a nucleic acid probe is added to the newly synthesized viral replicon RNA population which may comprise previously initiated RNAs as well as newly initiated RNAs. The nucleic acid probe comprises a region complementary at least a portion of the transcription initiation region of the viral replicon RNAs (e.g., the newly synthesized RNA population). When the probe is hybridized to the population of newly synthesized viral replicon RNAs, a portion of the transcription initiation region becomes double-stranded, with the remainder of the RNA being single-stranded. The single-stranded RNA may then be removed, for example, by digestion with a single-strand specific ribonuclease specific for single-stranded RNA. Suitable single-strand specific ribonucleases include, for example, ribonuclease T1, ribonuclease A, nuclease S1, and combinations comprising one or more of the foregoing single-strand specific ribonucleases. After digestion, the remaining RNA will thus be a double-stranded RNA (i.e., protected) population in which the previously initiated RNAs will comprise no labeled nucleotide analog, and the

newly initiated RNAs comprise the labeled nucleotide analog. The protected newly initiated RNAs can then be detected and/or quantified as described above.

[0060] Hybridization of the probe may be performed under conditions which are sufficient to allow specific hybridization of the nucleic acid probe to the viral replicase RNA. Specific hybridization can be performed under high stringency conditions or moderate stringency conditions, for example.

[0061] In a particularly preferred embodiment, the hybridization conditions for specific hybridization are high stringency.

[0062] The assay described herein may be employed to screen test compounds for inhibition of viral replicon RNA synthesis. Such compounds include replicase complex activity inhibitors as well as RNA synthesis initiation and elongation inhibitors. The method may be employed to distinguish initiation inhibitors from elongation inhibitors. If a viral replicon RNA synthesis inhibitor is added to a replication mixture, less viral replicon RNA should be synthesized, and the amount of labeled viral replicon RNA detected will be decreased (e.g., test RNA amount) relative to the amount of viral replicon RNA in a control sample (i.e., control RNA amount) with no added inhibitor. The amount of labeled viral replicon RNA detected will be decreased, for example, if the viral replicon RNA synthesis inhibitor is a viral replicon RNA elongation inhibitor. Viral replicon RNA elongation inhibitors will block synthesis of newly initiated viral replicon RNA elongation products and also previously initiated viral replicon RNA elongation products. If, however, the viral replicon RNA synthesis inhibitor is an initiation inhibitor, signal strength will be only modestly affected. Most (e.g., approximately 90%) of the newly synthesized viral RNAs of HCV are the elongation products of previously initiated viral replicon RNAs. Thus, only a small fraction (e.g., less than about 10%) of the newly synthesized replicon RNAs will be affected by an initiation inhibitor. FIGURE 1 provides a schematic of the inhibition of HCV RNA synthesis by initiation and elongation inhibitors.

[0063] An RNAse protection assay, as described herein, can be added after formation of the newly synthesized RNA population to identify initiation inhibitors. In this assay, a nucleic acid probe which is complementary to at least a portion of the initiation region of the newly synthesized viral replicon is employed. The probe is hybridized to the viral replicon RNA such that the region of the viral replicon RNA complementary to the probe is double-stranded (i.e., replicated), and the remainder of the viral replicon RNA is single-stranded.

Hybridization is preferably performed under stringent conditions. The single-stranded portion of the viral replicon RNA is then digested with a ribonuclease specific for single-stranded RNA such as, for example, ribonuclease A, ribonuclease T1, nuclease S1, or a combination comprising one or more of the foregoing ribonucleases. The remaining population of double-stranded replicon RNA will then contain two fractions: a fraction of previously initiated viral replicon RNAs which comprise no labeled nucleotide analog, and a fraction of viral replicon RNAs initiated after addition of the labeled nucleotide analog which comprise the labeled nucleotide analog. The protected viral replicon RNAs containing the labeled nucleotide analog can then be specifically detected and/or quantified as described above.

[0064] In the case of HCV, an exemplary probe is an RNA complimentary to nucleotides 15 to 433 of the HCV replicon RNA (SEQ ID NO:6), which hybridizes with the newly synthesized viral replicase RNA near the initiation codon. The RNA not hybridized to the probe is digested with a single-stranded ribonuclease, leaving only nucleotides 15 to 433 of the replicon RNA. The digested RNA will have two double-stranded RNA populations: a population of previously initiated replicon RNAs which do not contain the labeled nucleotide analog, and a population of newly initiated replicon RNAs which contain the labeled nucleotide analog. Thus, previously initiated HCV RNA elongation products will not be detected, while newly synthesized RNAs will be detected. In this assay including RNA protection, when an initiation inhibitor is added to the replication mixture, a reduced amount of labeled nucleotide analog is incorporated at the initiation site, and the signal measured by the means for detection will be decreased.

[0065] In positive strand RNA viruses, the replicase complex is a multi-protein complex which replicates viral replicon RNA from a viral replicon template RNA. Replicase complexes which include viral replicon template RNAs in addition to replication proteins can be isolated as a membrane bound fraction from cells expressing the viral replicon RNA. The isolated replicase complexes may include both previously initiated and uninitiated viral replicon template RNAs. Upon addition of nucleotide triphosphates and other components sufficient for RNA synthesis, elongation of previously initiated template RNAs proceeds to produce full-length viral replicase RNAs. It had not, however, been demonstrated that *de novo* initiation of uninitiated viral replicon template RNAs occurs in isolated replicase complexes. By using an inhibitor of initiation of viral replicon RNA synthesis initiation, it

can be shown that *de novo* initiation of viral replicase RNA synthesis occurs in isolated replicase complexes. Because *de novo* initiation of RNA synthesis takes place in the isolated replicase complexes, the methods may be used to quantify newly initiated RNA and also to identify inhibitors of RNA synthesis initiation.

[0066] Thus, the described methods are suitable for detecting inhibitors of RNA synthesis of positive strand RNA viruses such as RNA initiation and elongation inhibitors.

[0067] Also provided herein is a kit for screening a test compound for inhibition of RNA synthesis of a positive strand RNA virus. The kit comprises an isolated replicase complex for the positive strand RNA virus, and isolated viral replicon template RNA for the positive strand RNA virus, instructions for use of the kit, and buffers and nucleoside triphosphates which are sufficient for synthesis of the viral replicon RNA. Instructions may include, for example, instructions for quantitating a newly synthesized RNA population, instructions for determining if a compound is an RNA synthesis inhibitor, and the like. The instructions may be written instructions, although electronic storage media (e.g., magnetic diskette or optical disk) containing instructions are also acceptable, relating to the use of components of the methods. The instructions included with the kit may include information as to reagents (e.g., whether included or not in the kit) necessary for practicing the methods, instructions on how to use the kit, and/or appropriate reaction conditions.

[0068] Suitable buffers include those described previously as suitable for performing replication with isolated replication complexes.

[0069] The kits may optionally further comprise, for example, a labeled nucleotide analog, a means for detecting and/or quantitating a labeled nucleotide analog, a primer, a single-strand specific ribonuclease, and instructions for performing an RNAse protection assay.

[0070] The component(s) of the kit may be packaged in a convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations.

[0071] The invention is further illustrated by the following nonlimiting examples.

EXAMPLES

EXAMPLE 1. GROWTH AND MAINTENANCE OF HCV REPLICON CONTAINING CELLS

[0072] RNA molecules encoding the HCV replicon (i.e., viral replicon RNAs) are transfected into Huh-7 cells using electroporation.

[0073] The equipment and materials for cell maintenance include, but are not limited to, Huh-7 HCV replicon-containing cells, maintenance media (DMEM (Dulbecco's modified Eagle media) supplemented with 10% FBS (fetal bovine serum), L-glutamine, non-essential amino acids, penicillin (100 units/ml), streptomycin (100 micrograms/ml), and 500 micrograms/ml of Geneticin (G418), screening media (DMEM supplemented with 10% FBS, L-glutamine, and non-essential amino acid, penicillin (100 units/ml) and streptomycin (100 micrograms/ml)), 96 well tissue culture plates (flat bottom), 96 well plates (U bottom for drug dilution), Interferon alpha for positive control, fixation reagent (such as methanol: acetone), primary antibody (rabbit anti-NPTII), secondary antibody: Eu-N1 l, and enhancement solution.

[0074] Cells containing the HCV replicon support high levels of viral RNA replication when their density is suitable. Over-confluency may cause decreased viral RNA replication. Therefore, cells should be grown in log phase in the presence of 500 micrograms/ml of G418. Generally, cells should be passed twice a week at 1: 4-6 dilution. Cell maintenance is conducted as follows:

[0075] Cells containing the HCV replicon DNA vector are examined under a microscope to ensure that the cells are growing well. Cells are rinsed once with phosphate buffered saline (PBS) and 2 ml trypsin is added. The cell/ trypsin mixture is incubated at 37°C in a CO_2 incubator for 3-5 minutes. After incubation, 10 ml of complete media is added to stop the trypsinization reaction. Cells are blown gently, put into a 15 ml tube, and spun at 1200 rpm for 4 minutes. The trypsin/ medium solution is removed and the pelleted cells are recovered.

EXAMPLE 2. QUANTITATIVE ASSAY FOR DETECTION OF NEWLY SYNTHESIZED HCV RNA IN CELL-FREE SYSTEM

[0076] Membrane Fractions are purified from Huh-7 cells transfected with the HCV replicon RNA according to the procedure given by Hardy, et al. (*J. Virol.* (2003) 77:2029-2037). Briefly, the HCV replicon-containing cells are washed with 1X PBS, re-suspended in cold hypotonic buffer (10mM Tri-HCl, pH 7.8, 10 mM NaCl), and put on ice for 20 minutes. The swelled cells are disrupted using a dounce homogenizer. The mix is centrifuged at 900 X g for 5 minutes at 4°C. The supernatant is transferred to a fresh tube and centrifuged at

15000 X g for 25 minutes at 4°C. The pellet, which contains the membrane fraction, is re-suspended in storage buffer (hypotonic buffer with 15% glycerol), and may be stored at – 80°C.

[0077] *In vitro* RNA replication is performed according to the procedure of Lai (*J. Virol.* (2003) 77:2295-2300) with some modifications. Test compound (1 μM to 100 μM in 0.5 μl DMSO) is pre-incubated the membrane fraction (isolated replicase complexes and isolated viral replicon template RNA) (10 μl) for 10 minutes at 30°C before the replication mix is added. The replication mix (50 μl total volume) contains 50 mM HEPES (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$ 50 U RNAsin, 10 μg/ ml actinomycin D, 2.5 mM ATP, 0.5 mM each CTP, GTP, Br-UTP and the pre-incubated membrane fraction (i.e., the replicase complex and viral replicon template RNA). The replication mix is incubated at 30°C for 2 hours, followed by purification using TRIZOL (Invitrogen, Carlsbad, CA) or RNA EASY (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA samples are then immuno-precipitated using anti-BrdU monoclonal antibody (Molecular Probes, Eugene, OR) and protein A agarose (Invitrogen) in 200 μl to 400 μl of precipitation buffer containing 1X PBS, 0.05% NP 40, 0.1 μg/ μl tRNA, 5 U/ μl RNasin, and 0.3 M uridine.

[0078] When the newly synthesized segment near the initiation site is of interest, an RNA protection assay is used after Br-UTP labeling and RNA purification. The RNA probe for segment from 15 to 433 nt (SEQ ID NO: 6) is synthesized using an *in vitro* transcription kit (MAXIscript, Ambion). The DNA template for *in vitro* transcription was made by PCR using forward primer 5' GGGGGCGACACTCCACCATAGAT (15-37) (SEQ ID NO: 7) and reverse primer 5' ATTTAGGTGACACTATAGAAACCCAAGCGGCCGGAGAACCT (413-433, plus SP6 core sequence) (SEQ ID NO:8) on DNA vector for replicon HCV RNA or cDNA made from replicon HCV RNA by reverse transcription. The RNA is protected using an RNA protect assay kit (e.g. RPA III, Ambion, Austin, TX). Briefly, in the RNA protection assay, the newly synthesized replicon RNAs are hybridized to the RNA probe. The hybridized RNAs are then treated with a mixture of ribonuclease A and ribonuclease T1 to digest the single-stranded RNA. The remaining double-stranded RNA may then be precipitated prior to detecting.

[0079] The newly synthesized HCV RNA produced in the cell-free system is quantitatively detected using real time PCR. Table 1 shows the data for several control experiments. A Taqman® probe labeled with fluorescent dye FAM was used.

TABLE 1

Sample	Copy number after PCR with no immuno-precipitation with anti-BrdUTP antibody	Copy number after PCR performed after immuno-precipitation with anti-BrdUTP antibody
Template RNA isolated from Huh-7 cells expressing HCV replicon with Br-UTP	1.86×10^7	2.05×10^5
Template RNA isolated from Huh-7 cells expressing HCV replicon with Br-UTP: No anti-Brd-UTP antibody	1.85×10^7	2401
Membranes isolated from Huh-7 cells with no HCV (no HCV template RNA)	15	3
Template RNA isolated from Huh-7 cells expressing HCV replicon: No Br-UTP in replication	1.79×10^7	7270

[0080] As seen from Table 1, for template RNA isolated from Huh-7 cells expressing the HCV replicon with Br-UTP in the replication mixture, a high copy number of replicon RNA is observed both with and without precipitation with an anti-BrdUTP antibody. If no anti-BrdUTP antibody is used, the sample without precipitation has a high copy number of HCV replicon RNA, while the precipitated population has only a background amount. If the Huh-7 cells are not transfected with the HCV replicon (i.e., no template RNA), a background amount of replicon RNA is observed with or without precipitation with an anti-BrdU

antibody. If there is no Br-UTP in the replication mixture with template RNA isolated from Huh-7 cells expressing the HCV replicon, the sample without precipitation has a high copy number of HCV replicon RNA, while the precipitated population has only a background amount. Thus, the control experiments give the expected results.

[0081] As shown in Table 2, the copy number of the HCV RNA measured by quantitative PCR is dependent upon the amount of template RNA added to the replication mixture. In Table 2, HCV is a replication mixture with template RNA isolated from Huh-7 cells expressing the HCV replicon. HVC (1/2) and HCV (1/8) are replication mixtures containing ½ and 1/8 of the amount of template RNA, respectively. Huh-7 (no HCV) is a no HCV template control as described above. No Br-UTP is a control in which template RNA isolated from Huh-7 cells expressing the HCV replicon, but no Br-UTP is added to the replication mixture.

TABLE 2

Sample	Copy number after PCR with no immuno-precipitation with anti-BrdUTP antibody	Copy number after PCR performed after immuno-precipitation with anti-BrdUTP antibody
HCV	2.19×10^7	7.34×10^5
HCV (1/2)	1×10^7	3.7×10^5
HCV (1/8)	3.1×10^6	1.51×10^5
Huh-7 (No HCV)	19	2030
No Br-UTP	2.17×10^7	4.6×10^4

[0082] As shown in Table 2, the copy number of HCV replicons measured in the assay, both with and without precipitation with an anti-BrdUTP antibody, is directly proportional to the amount of template replicon RNA added to the reaction mixture.

[0083] Table 3 shows the data for a known HCV RNA replication inhibitor that is tested using the disclosed assay at a concentration of 10 μM. The test compound is a published compound (Dhanak et al., *J. Biol. Chem.* (2002) 41):38322-7. The structure of the test compound is shown below.

4

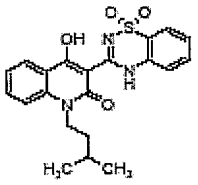

TABLE 3

Sample	Copy number after PCR with no RNAse protection assay		Copy number after PCR with RNAse protection assay	
		Copies as % of samples with no test compound		Copies as % of samples with no test compound
No test compound	46942	100	137494	100
Test compound	31156	66	16970	12

[0084] In Table 3, although treatment with the test compound results in less than half reduction in total viral RNA synthesis with no RNAse protection, it causes close to a 10-fold reduction in the amount of newly initiated viral RNA synthesis after performing RNAse protection. Thus, the test compound inhibits HCV RNA synthesis at the initiation stage.

[0085] The results reported in this example validate this assay for compound screening and profiling. The assay has the advantage of quantitatively detecting newly synthesized HCV RNA, and permitting but also specific segment of our choice.

[0086] Example 3. Use of an RNA initiation inhibitor to demonstrate *de novo* initiation of replication in isolated replication complexes

[0087] An RNA replication assay was used to determine if the replication complexes isolated from a membrane fraction were enzymatically active. The test compound (an initiation inhibitor) was employed to determine if the isolated replication complexes were capable of *de novo* initiation of newly synthesized RNA. Standard replication mixtures contained 50 mM HEPES (pH 7.3); 10 mM KCl; 10 mM $MgCl_2$; 0.3 mM $MnCl_2$; 20 Units of RNAse inhibitor; 10 μg of actinimycin D per mL; 0.5 mM ATP, GTP, and UTP; 10 μCi of [α-P^{32}] CTP; and 6 μl of the membrane fraction in a total volume of 60 μl. The reaction mixtures were incubated at 30°C for 2 hours. The RNA products were extracted with phenol-chloroform, ethanol precipitated, and separated on a non-denaturing 1% agarose gel. After electrophoresis, the gel was fixed with 10% glacial acetic acid then in ethanol and dried prior to autoradiography.

[0088] Figure 2 shows an autoradiogram of the experimental results. Lane 4 is a control lane with no added inhibitor. Lanes 1-3 show the results with initiation inhibitor concentrations of 100, 20, and 4 μM, respectively. As clearly shown in the autoradiogram, addition of the initiation inhibitor reduces the amount of single-stranded RNA produced by the replication complex. In the assay, double-stranded RNA is produced largely from elongation of previously initiated RNAs, while single-stranded RNA is produced from newly initiated RNAs. The presence of a single-stranded RNA band in control lane 4 shows that newly initiated RNAs are formed during replication. When the test compound, a known RNA synthesis initiation inhibitor, is added during replication, the single-stranded RNA band disappears, while the double-stranded RNA band remains. Thus, by employing an RNA synthesis initiation inhibitor, it is confirmed that *de novo* RNA synthesis initiation occurs in isolated replicase complexes.

[0089] A method has been described to identify inhibitors of viral replicon RNA synthesis using detection and/or quantitation of a population of newly synthesized RNA. A decrease in the amount of newly synthesized RNA made in the presence of a test compound compared to a control with no test compound indicates that the test compound is a viral replicon RNA synthesis inhibitor. The method is particularly useful for identifying RNA elongation inhibitors. In addition, an RNAse protection assay may be employed on the newly

synthesized RNA to identify RNA initiation inhibitors. It has been clearly demonstrated that isolated replication complexes are capable of *de novo* initiation in addition to elongation of previously initiated RNA. An advantage of the method is that initiation inhibitors can be distinguished from elongation inhibitors. Another advantage is that quantitation of the newly synthesized RNA population is employed to identify viral replicon RNA synthesis inhibitors. Another advantage is that a 2'-O-methylated nucleotide can be used to increase the yield of newly synthesized RNA produced during replication.

[0090] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.